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Rapid determination of endogenous cytokinins in plant samples by combination of magnetic solid phase extraction with hydrophilic interaction chromatography–tandem mass spectrometry

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ABSTRACT

A 2-acrylamido-2-methyl-1-propanesulfonic acid-co-ethylene glycol dimethacrylate (Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)) copolymer was prepared and used as a magnetic solid phase extraction (MSPE) medium for recovery of endogenous cytokinins (CKs) from plant extracts. This magnetic porous polymer was characterized by electron microscopy, nitrogen sorption experiments, elemental analysis and Fourier-transformed infrared spectroscopy. It was demonstrated to have high extraction capacity toward CKs in plants due to its specificity, surface area and porous structure. Coupled with hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS), a rapid, simple, and effective MSPE–HILIC–MS/MS analytical method for the quantitative analysis of endogenous CKs in *Oryza sativa* (*O. sativa*) roots was successfully established. Good linearities were obtained for all CKs investigated with correlation coefficients (R^2) > 0.9975. The results showed that LODs (S/N = 3) were ranged from 0.18 to 3.65 pg mL^{-1}. Reproducibility of the method was obtained with intra-day and inter-day relative standard deviations (RSDs) less than 16.1% and the recoveries in plant samples ranged from 72.8% to 115.5%. Finally, the MSPE–HILIC–MS/MS method was applied to several plant samples, and the amounts of endogenous CKs in *O. sativa* roots, leaves and *Arabidopsis thaliana* (*A. thaliana*) were successfully determined.

1. Introduction

Naturally occurring cytokinins, adenine-derived signalling molecules with an isoprenoid or aromatic side chain, are a group of plant hormones which regulate a wide variety of physiological and developmental processes. Since their discovered in the 1950s [1], more than 40 cytokinins have been identified in plant tissues [2] and their functions on plant growth and development such as cell division and differentiation [3], formation and growth of roots and shoots [4], regeneration of root damage [5], apical dominance and senescence [6] have been investigated.

To complete a detailed study of the function, metabolism, and signal transduction of CKs in plants and other organisms, quantification of their endogenous levels is required. Nowadays, many techniques have been developed for quantification of CKs, such as gas chromatography (GC) [7], high performance liquid chromatography (HPLC) [8–10], ultra-high performance liquid chromatography (UHPLC) [11] and capillary electrophoresis (CE) [12] coupled to mass spectrometry (MS). Among these techniques, HPLC–MS, especially reversed-phase liquid chromatography with MS (RPLC–MS) is the most commonly used technique because of its high resolution separation, high sensitivity and unequivocal identification of target analytes. Recently, we introduced hydrophilic interaction chromatography coupled to MS (HILIC–MS) into CKs analysis [9], which greatly improved the detection sensitivity compared with traditional RPLC–MS.

The extremely low concentration levels of CKs in plant tissues (pmol g^{-1} fresh weight) [13] and the complex plant matrix extracts make it more difficult to determine CKs in plant samples accurately. Thus, sample enrichment and purification techniques are required prior to chromatography. Up to now, many sample pretreatment techniques have been employed for extraction, pre-concentration and purification of CKs from plants, such as immunoaffinity purification [11,13], liquid–liquid extraction (LLE) [14], solid phase extraction (SPE) [8,15,16] and polymer monolith microextraction (PMME) [9]. In each case, the procedure requires the combination of two or more steps. Among these techniques, SPE with C_{18} and mixed-mode polymeric strong cation exchange and reversed-phase materials (MCX) as sorbents [15] has been the most commonly used method due to its powerful preconcentration and purification ability and the flexibility of available commercial adsorbents. However, the traditional SPE procedure is relatively

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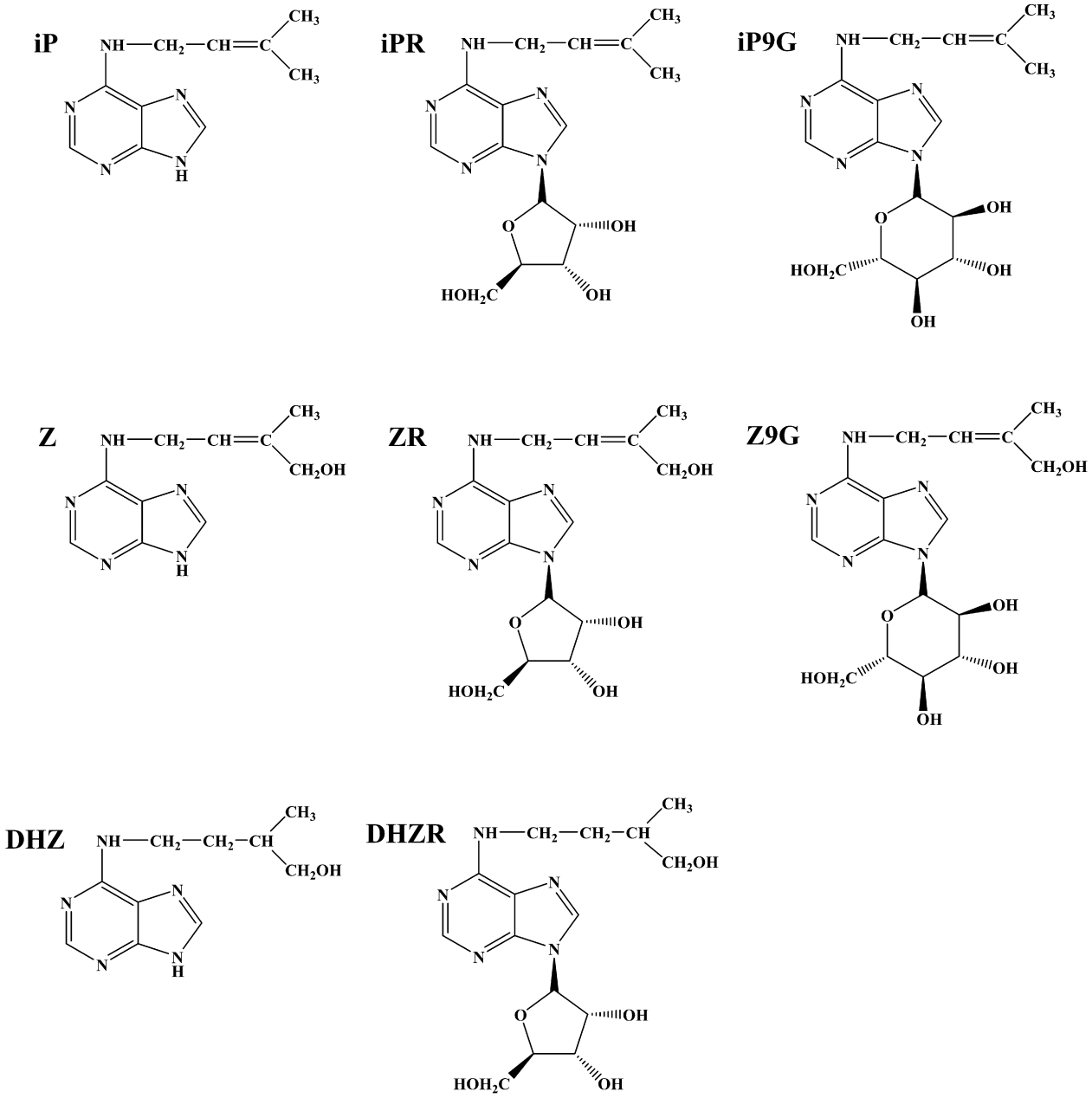

Fig. 1. Structures of target cytokinins.

tedious and time consuming [17]. Recently, our group developed a tandem SPE-online trapping method, which required only 20 mg plant tissues for quantification of plant CKs [18].

In recent years, magnetic solid-phase extraction (MSPE) has received much attention in sample pretreatment [19–23]. The technique is based on magnetic or magnetizable adsorbents with high adsorption ability and superparamagnetism. Different from the traditional SPE, the magnetic adsorbents do not need to be packed into a SPE cartridge but are dispersed uniformly in a sample solution or suspension. Like other dispersive SPE methods, column blocking during extraction is avoided but the sorbent can be readily isolated from the sample matrix with a magnetic lure. In addition, this mode can facilitate mass transfer of analytes by drastically increasing the interfacial area between the solid adsorbent and sample solution [19], thus reducing the extraction time.

Hitherto, many studies have reported the application of MSPE in fields of food analysis [19,21], environmental analysis [23–26], and biological analysis [22] with Fe_3O_4 particles modified with hydrophobic groups [26], hydrophilic groups [27], ion exchange groups [28], mixed mode groups [23] or even metal ions [29]. However, to our best knowledge, no application of MSPE has been reported to the analysis of CKs in complex plant tissues.

In the current study, a new magnetic porous polymer, Fe_3O_4/SiO_2/poly (2-acrylamido-2-methyl-1-propanesulfonic acid-co-ethylene glycol dimethacrylate) (Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)), was prepared for purification and enrichment of CKs (Fig. 1) in plant roots and leaves. The sorbent has a large surface area and is highly specific giving satisfactory adsorption capacity and fast mass transfer. Thus, in current study, we developed a MSPE method combined with hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) for the determination of CKs in plant tissues. The versatility of the method was demonstrated for the analysis of CKs in *Oryza sativa*

(*O. sativa*) roots and leaves and *Arabidopsis thaliana* (*A. thaliana*) seedlings.

2. Experimental

2.1. Chemicals and reagents

Ferric chloride ($FeCl_3{\cdot}6H_2O$), sodium acetate (NaAc), ethylene glycol (EG), 1,2-ethylenediamine (ETH), ethanol (EtOH), azobisisobutyronitrile (AIBN) and 2-acrylamido-2-methyl-1-propanesulfonic acid-co-ethylene dimethacrylate (AMPS, 99% pure) were all purchased from Sinopharm Chemical Reagent (Shanghai, China). Ethylene dimethacrylate (EGDMA, 98% pure) were purchased from Acros (New Jersey, USA). Tetraethylorthosilicate (TEOS), 3-(methacryloxy)propyl trimethoxysilane (MPS) were bought from Wuhan University Silicone New Material (Wuhan, China). AIBN was recrystallized from ethanol immediately prior to use. Other reagents were used as received without further purification.

Acetonitrile (ACN, HPLC grade) was obtained from Tedia company (Fairfield, OH, USA). Methanol (MeOH, HPLC grade) was obtained from J&K Chemical (Beijing, China). Acetone (HPLC grade) was purchased from Fisher Company Inc. (Fairfield, Ohio, USA). Formic acid (LCMS grade) and ammonium hydroxide solution (25 wt%; LCMS grade) were supplied by Fluka (Steinheim, Germany). Purified water was obtained from a Waters Milli-Q system (Milford, MA, USA) and was used for all aqueous solutions. HiCapt SPE C_{18} cartridges were purchased from Weltech (Wuhan, China).

Cytokinins: N^6-(-isopentenyl)adenine (iP), isopentenyladenine riboside (iPR), isopentenyladenine riboside 9-glucoside (iP9G), trans-zeatin (t-Z), trans-zeatin-riboside (t-ZR), trans-zeatin 9-glucoside (t-Z9G), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), and stable isotope-labeled standards: [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_5$]t-Z, [$^2H_5$]t-ZR, [$^2H_5$]t-Z9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR were purchased from Olchemim (Olomouc, Czech Republic).

2.2. Plant materials

O. sativa and *A. thaliana* seeds were grown in green house at 33 °C under 16 h photoperiods. Seven-day-old *O. sativa* roots and leaves and one month *A. thaliana* seedlings were harvested, weighted, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Preparation of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)

Fe_3O_4 nanoparticles were synthetized according to a previously reported procedure with minor modifications [30]. In brief, $FeCl_3{\cdot}6H_2O$ (5 g) was dissolved in EG (100 mL) to form a clear solution, followed by the addition of NaAc (15 g) and ETH (50 mL). The mixture was stirred vigorously for 30 min and then sealed in a Teflon lined stainless-steel autoclave. The autoclave was heated to and maintained at 200 °C for 8 h then allowed to cool to room temperature. The resultant Fe_3O_4 nanoparticles were washed with water and methanol repeatedly with the aid of a magnet and then dried in a vacuum oven at 60 °C.

The synthetic Fe_3O_4 nanoparticles (600 mg) were suspended in H_2O (30 mL) and well dispersed with a model SB-5200D ultrasonic mixer (Ningbo Scientz Biotechnology Co. LTD., Ningbo, China). EtOH (934 mL), H_2O (266 mL), ammonium hydroxide solution (30 mL) and TEOS (24 mL) were successively added into a 2000 mL three-necked round bottom flask, and then the dispersed Fe_3O_4 nanoparticles were added into the mixture. With a mechanical stirring, the reaction system was kept at room temperature for 12 h. The resulting Fe_3O_4/SiO_2 particles were washed sequentially with water and methanol. A magnetic lure was used to recover the particles and they were then dried in a vacuum oven at 60 °C.

Magnetic silica colloids with surface modification by MPS were synthesized as follows. Fe_3O_4/SiO_2 particles (1.2 g) were suspended in anhydrous toluene (500 mL) with mechanical stirring, then MPS (1.2 g) was introduced into the solution and the mixture was stirred for 24 h at room temperature. The resultant MPS-modified Fe_3O_4/SiO_2 was washed sequentially with water and methanol, recovered with the magnetic lure and then dried in a vacuum oven at 60 °C.

The Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) composite was synthesized by the distillation–precipitation polymerization method as proposed by Yang et al. [31], wherein the monomer (AMPS), cross-linker (EGDMA) and initiator (AIBN) proportion of P(AMPS-co-EGDMA) was added to the reaction system according to our previous report [9,32,33]. MPS-modified Fe_3O_4/SiO_2 (0.5 g), AMPS (1.2 g), EGDMA (8.4 mL), AIBN (0.120 g), and acetonitrile (100 mL) were successively added into a 500-mL two-necked round bottom flask equipped with a distillation apparatus and a stirring device. The mixture was heated to boiling within 30 min and was kept at the boil until approximately half of the acetonitrile was distilled out (occurring within 2 h). The mixture was then cooled to room temperature, the resultant Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) was separated by the aid of a magnet, and then washed several times with water and methanol. Finally, the resultant magnetic polymer was dried under vacuum at 60 °C.

2.4. Sample preparation

As shown in Fig. 2, plant tissues (*O. sativa* roots, leaves and *A. thaliana* seedlings (200 mg of fresh weight)) were frozen with liquid nitrogen, ground into powder with a mortar and pestle and then transferred into a 2-mL centrifuge tube. The stable isotope labeled CKs were added to each of the samples (0.4 ng of each compound per sample) to serve as internal standards for the quantification, then 1.2 mL modified Bieleski solvent (methanol/water/formic acid, 15/4/1, v/v/v) was added into the mixture. After extraction overnight at −20 °C, solids were separated by freeze centrifugation (Shanghai anting scientific instruement factory, Shanghai, China) (10,000 rpm, 20 min) at 0 °C. Supernatants liquids were passed through a C_{18} SPE cartridge (50 mg) which was preconditioned with 2 mL methanol and 2 mL modified Bieleski solvent to remove lipids and pigments. The unretained fraction was collected and diluted with 10 mL ammonium formate (5 mM, pH 3). After that, magnetic solid-phase extraction was carried out. Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) (50 mg) was put into a 15-mL vial and activated with 3 mL methanol and ammonium formate (5 mM, pH 3) in sequence, and then the diluted plant extracts were added into the vial. The mixture was vortexed for 1 min to form a homogeneous dispersion and then the supernatant liquid was separated and discarded by placing a magnet on the outer wall of the vial. After that, the collected magnetic sorbent was washed with water (1 mL) in a vortex for 30 seconds and recovered with the magnetic lure. Subsequently, 3 mL of acetone with 5% ammonia (v/v) was added to the sorbent to elute out the CKs, the eluent was separated and evaporated to dryness under a mild nitrogen stream at 35 °C. The residues were redissolving in 100 μL of mobile phase and 10 μL was then injected into the HILIC–MS/MS system for the analysis of CKs.

2.5. Instruments and analytical conditions

Scanning electron microscopy images were taken using JSM-6700F field emission scanning electron microscope (FESEM, JEOL, Japan). Nitrogen sorption experiments were carried out at 77 K using a JW-BK surface area and pore size analyzer (JWGB Sci. &

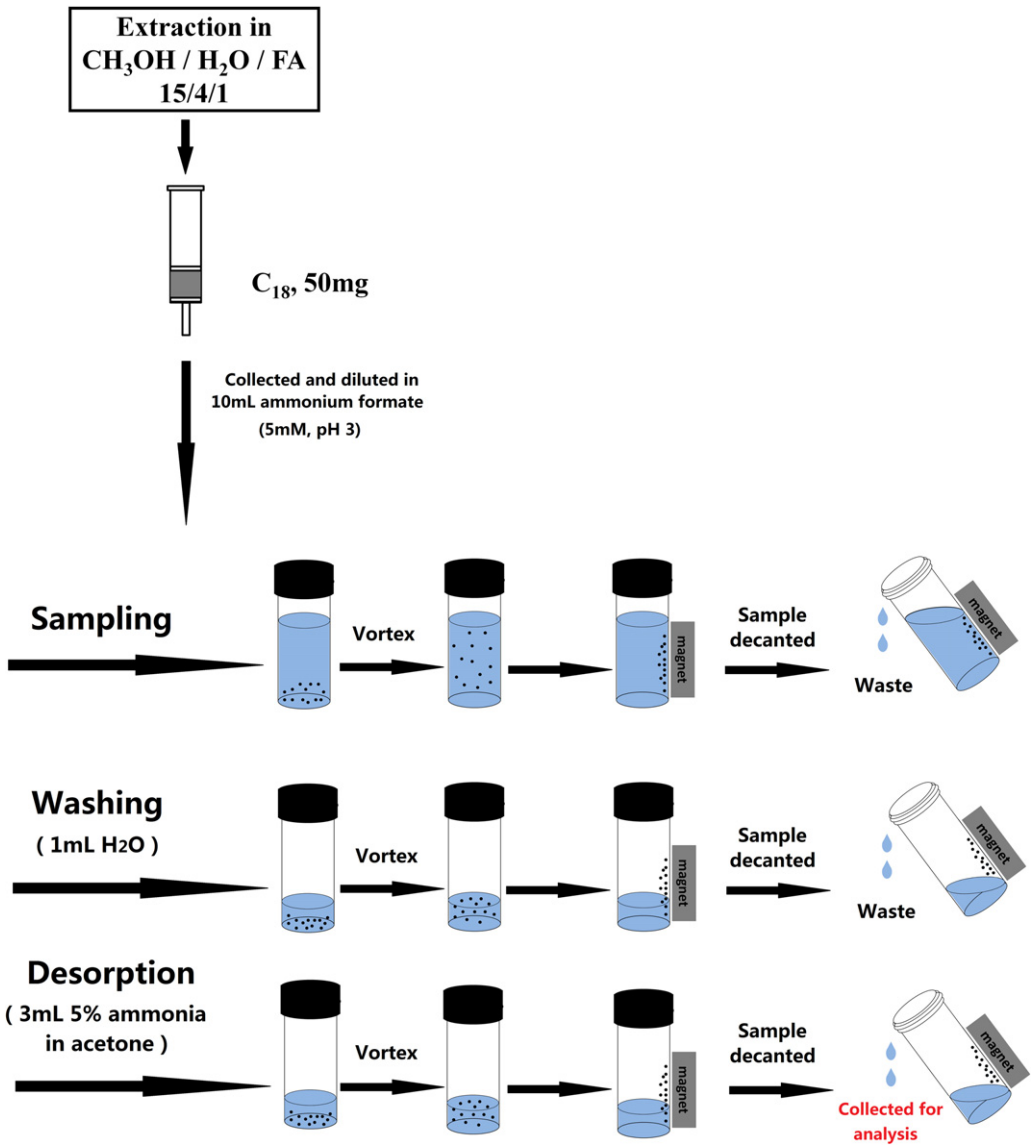

Fig. 2. Magnetic solid phase extraction protocol for cytokinins.

Tech., Beijing, China). Elemental analysis was performed on an Elementar VarioEL III elemental analyzer (Hanau, Germany). FT-IR spectra were obtained with a Thermo Nicolet 670 FT-IR instrument (Thermo, USA).

Analysis of CKs was performed on a HPLC-ESI (+)-MS/MS system consisting of a Shimadzu LC-20AD HPLC system (Tokyo, Japan) with two LC-20AD pumps, a SIL-20A auto sampler, a CTO-20AC thermostated column compartment, a DGU-20A3 degasse, and a AB SCIEX 3200 QTRAP MS/MS (Foster City, CA, USA) with an electrospray ionization source (Turbo Ionspray). Data acquisition and processing were performed using AB SCIEX Analyst 1.5 software (Foster City, CA, USA).

The HPLC separation was performed on a Luna Silica column (250 mm × 2.0 mm i.d., 5 μm) purchased from Phenomenex (Torrance, CA, USA) at 35 °C in HILIC mode. ACN and water (85:15, v/v) containing 0.01% FA (v/v) was used as mobile phase. The flow rate was set to 0.2 mL min^{-1}.

Multiple reaction monitoring (MRM) of $[M+H]^+$ and the appropriate product ions were chosen to quantify the target analytes. The optimized conditions for selective MRM experiments were the same as our previous study [9].

2.6. Method validation

To evaluate the linearity of the method, standard solutions containing increasing concentration of CK standards of 0.1, 0.3, 0.5, 1, 5, 10, 20, 50, 100 ng mL^{-1} and fixed amounts of corresponding internal standards (0.4 ng in 100 μL mobile phase) were used to create calibration curves from triplicate analyses of CK standards by plotting the peak area (analyte/IS) against the CKs concentrations for eight CKs. The limits of detection (LOD) and limits of quantification (LOQ) were determined at a concentration where the S:N ratios were equal to 3 and 10, respectively. Linearity was evaluated by the correlation coefficients (R^2) of the calibration curves.

The method reproducibility and accuracy were determined as follows. Plant extracts were divided into several aliquots and each aliquot was corresponded to 200 mg fresh weight of *O. sativa* roots. Aliquots of the extracts were spiked with three different

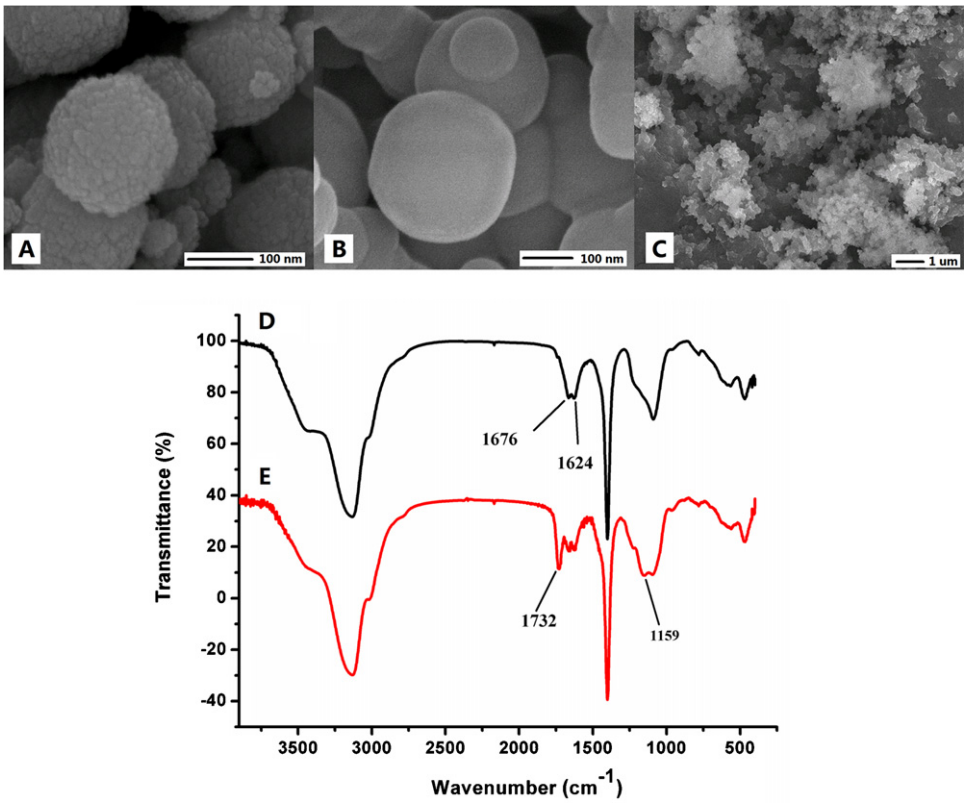

Fig. 3. FESEM images of Fe_3O_4 (A), Fe_3O_4/SiO_2 (B), and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) (C) magnetic materials; FT-IR spectra of the MPS-modified Fe_3O_4/SiO_2 (D) and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) (E).

concentrations of CK standards (0.5, 5 and 50 ng g^{-1}) and the same amounts of internal standards (2 ng g^{-1}) and then extracted using MSPE procedure and analyzed by HILIC–MS/MS. The intraday variation was evaluated by repeating the process for three times within one day, and the interday variation was investigated on three successive days. The accuracy was measured by the recovery of the whole method at the three spiked concentrations according to the calibration curves.

3. Results and discussion

3.1. Characterization of magnetic polymer Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)

The morphology of the resulting products (particles of Fe_3O_4, Fe_3O_4/SiO_2 and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)) were investigated by FESEM. Fig. 3A shows the typical SEM image of the Fe_3O_4 NPs. Clearly, these Fe_3O_4 NPs are monodisperse in size and have spherical morphology with rough surfaces and a mean size around 100 nm. As seen in Fig. 3B, after modification with TEOS, the particles (Fe_3O_4/SiO_2) still possess a spherical shape but with smooth surfaces and a mean diameter around 150 nm, indicating that silica is successfully coated on the surface. Fig. 3C provides the TEM image of the final product Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) particles. It can be seen that the magnetic polymer particles are of porous structure and formed clusters with diameter between 1 and 2 μm.

To further confirm the porous structures of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) particles, the specific surface were characterized by N_2 adsorption/desorption. The results show that the magnetic particles after polymerization yield desorption average pore diameter of 6.7 nm and larger specific surface of 106 m^2 g^{-1} than that of the particles before polymerization (8 m^2 g^{-1}), indicating that the final product Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) particles have a porous structure, which was concordant with the TEM results.

Element analysis was further employed to confirm the polymer layer of the final magnetic materials. As shown in Table 1, the C, H and S contents of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) are obviously higher than those of MPS-modified Fe_3O_4/SiO_2, indicating the successful coating of polymer on Fe_3O_4/SiO_2.

To further explore the surface composition, the FT-IR spectroscopy of MPS modified Fe_3O_4/SiO_2 and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) is shown in Fig. 3D and E, The MPS modification of Fe_3O_4/SiO_2 is supported by the IR bands at 1624 cm^{-1} and 1676 cm^{-1}, which are corresponding to the stretching vibration of vinyl and carbonyl moieties of MPS, respectively (Fig. 3D). As shown in Fig. 3E, the successful coating of P(AMPS-co-EGDMA) onto the surface of MPS-modified Fe_3O_4/SiO_2 is also demonstrated by the FT-IR spectrum with the presence of strong characteristic absorption bands at 1732 cm^{-1} and 1159 cm^{-1} attributable to the stretching vibration of the ester and sulfonate groups.

3.2. Reproducibility of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)

As an extraction medium, we recognize that sorbent reproducibility is an important factor which ensures its robustness and practicability. In this study, three batches of the magnetic polymer sorbents were synthesized and the intra- and inter-batch reproducibility was assessed by calculating the RSDs of the extraction efficiency while performing the extractions of CKs from standard samples. All the RSDs of intra-batch were in the range of 1.8–9.2% and inter-batch were 1.1–10.5% respectively, which confirmed that laboratory-made sorbents presented good reproducibility (Table 2).

Table 1
Elemental analysis of magnetic materials before and after polymerization.

Magnetic materials	C (wt %)	S (wt %)	H (wt %)	N (wt %)
MPS-modified Fe_3O_4/SiO_2	1.712	0.474	0.379	0.095
Fe_3O_4/SiO_2/P(AMPS-co-EGDMA)	35.88	2.331	4.738	0.282

Table 2
The RSD value of CKs with intra- and inter-batches of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) sorbent.

Precision (RSD, %)	iP	iPR	iP9G	Z	ZR	Z9G	DHZ	DHZR
Intra-batch (3)	9.2	4.5	1.8	2.2	3.0	4.9	4.1	4.0
Inter-batch(3)	10.5	3.7	1.2	4.6	9.5	1.1	4.4	7.4

3.3. Optimization of MSPE conditions

In order to maximize the extraction efficiency of the adsorbent towards CKs, various parameters including amount of the sorbent, pH and organic content of the sample matrix, extraction time, desorption solvent and desorption time needed to be optimized.

To evaluate the effect of the sorbent amount on extraction efficiency, different amount of 10, 20, 50, 70, 100 mg of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) sorbent were tested and the result showed that, the recoveries of CKs reached a maximum by 50 mg and greater amount of sorbent (Fig. 4). Thus, 50 mg was employed in the following experiment.

The pH of the sample matrix will affect the ionic state of CKs and therefore the extraction efficiency is predictably pH-dependent. Optimization was performed by using ammonium formate solutions in the pH range from 2 to 7 as the sample matrix for MSPE. As shown in Fig. 5A, the highest extraction efficiency for CKs is obtained in the pH range of 2.5–3 and an obviously decrease was found when the pH increased to 7. This result can be explained by the fact that the exocyclic amino group at the sixth position of purine in the structure of CKs has a pK_a 4, and so for a pH≤3, the CKs are all positively charged [15] and can interact with the polymer acidic pendant groups via strong cation exchange. With the increase of pH in the sample matrix, a weakened ion-exchange interaction yields a lower extraction efficiency. In the following experiment, matrix pH was adjusted to 3 with ammonium formate solution (5 mM).

Organic content in sample solution may also affect the extraction efficiency because of hydrophobic interactions between the analyte and the polymer backbone of magnetic sorbents. In our study, optimization was performed by using ammonium formate solutions with a methanol content from 0 to 70% as the sample matrix for MSPE. As shown in Fig. 5B, there was no obvious variation of extraction efficiency in the range of 0–10% for most CKs. With the incremental increase of organic content, extraction efficiency decreased sharply as methanol content increased. When dealing with plant samples, the extraction solvent from the C_{18} cartridge can be diluted directly in a suitable aqueous solvent to reduce the methanol content without the need for evaporation and redissolution. Thus, 10 mL ammonium formate buffer (5 mM) was used to dilute the plant matrix to ensure the methanol content in sample matrix did not exceeding 10%.

The effect of extraction time and desorption time was optimized by increasing the vortex time from 30 s to 15 min and 30 s to 10 min, respectively. The result showed that extraction time had no significant influence on the extraction efficiency. Therefore, 1 min was selected as the extraction time. In addition, 1 min was also enough to elute the extracted CKs from the Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) sorbents.

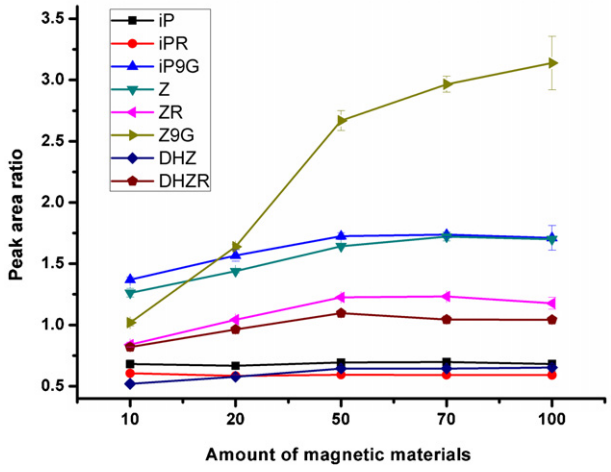

Fig. 4. Investigation of the amount of Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) adsorbent. Cytokinins standards were spiked in sample solutions at 1 ng mL^{-1}.

3.4. Comparison experiment

Under the optimized conditions, the comparison of extraction efficiency towards CKs for the Fe_3O_4/SiO_2 microspheres with and without the P(AMPS-co-EGDMA) layer were investigated to illustrate the influence of polymer layer. Fig. 6 displays the MRM chromatograms of direct injection of 1 ng mL^{-1} eight CKs and CKs extracted by MPS-modified Fe_3O_4/SiO_2 and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA), respectively. Obviously, much higher responses of CKs were gained from the magnetic polymer sorbent than that from MPS-modified Fe_3O_4/SiO_2 and direct injection, indicating the successfully coating of the polymer and its remarkable preconcentration ability towards the testing analytes. The extraction efficiencies were found to be 53.2–87.0% in standard samples and 13.9–70.8% in *O. sativa* root extracts spiked with CKs standards (Table 3).

3.5. Analytical performance

Calibration curves were created from triplicate analyses of CK standards by plotting the peak area (analyte/IS) against the CKs concentrations ranging from 0.1 to 100 ng mL^{-1}. As listed in Table 4, good linear correlation were obtained for all CKs with correlation coefficients (R^2) ranging from 0.9975 to 0.9998. LODs and LOQs were calculated as the signal-to-noise ratios of 3:1 and 10:1, respectively. The results showed that LODs and LOQs for eight CKs were ranged from 0.18 to 3.65 pg mL^{-1} and 0.59 to 12.20 pg mL^{-1}, respectively.

The accuracy of the method was measured and expressed as recovery. The precision of the method was assessed by determining intra- and inter-day RSDs of the analysis. Both recoveries and intra- and inter-day RSDs were calculated with eight CKs spiked in *O. sativa* root samples at three different concentrations (0.5, 5

Table 3
The extraction efficiency (%) of CKs extracted by Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) sorbent.

	iP	iPR	iP9G	Z	ZR	Z9G	DHZ
Standard sample	78.6	73.2	87.0	81.0	62.2	53.2	70.4
Real sample	70.8	56.7	38.5	40.0	29.1	13.9	39.5

Table 4
Calibration curves, LOD and LOQ data of CKs.

Analytes	Linear dynamic range (ng mL^{-1})	Regression line			LOD (pg mL^{-1})	LOQ (pg mL^{-1})
		Slope	Intercept	R^2 value		
iP	0.1–100	0.1420	−0.0570	0.9995	2.86	9.52
iPR	0.1–100	0.1140	−0.0279	0.9975	0.83	2.76
iP9G	0.1–100	0.3397	−0.2486	0.9990	1.12	3.73
Z	0.1–100	0.3491	−0.0750	0.9998	2.03	6.76
ZR	0.1–100	0.2928	−0.2077	0.9983	0.82	2.74
Z9G	0.1–100	0.9352	−0.2320	0.9991	0.18	0.59
DHZ	0.1–100	0.1269	−0.0104	0.9996	3.65	12.20
DHZR	0.1–100	0.1998	−0.0384	0.9989	0.54	1.80

Table 5
Precisions (intra- and inter-day) and recoveries for the determination of CKs in *O. sativa* roots.

Analytes	Intraday precision (R.S.D., %; $n = 5$)			Interday precision (R.S.D., %; $n = 3$)			Recovery (%)		
	Low (0.5 ng g^{-1})	Medium (5 ng g^{-1})	High (50 ng g^{-1})	Low (0.5 ng g^{-1})	Medium (5 ng g^{-1})	High (50 ng g^{-1})	Low (0.5 ng g^{-1})	Medium (5 ng g^{-1})	High (50 ng g^{-1})
iP	4.7	4.4	6.5	3.4	2.1	4.7	106.6	96.4	92.9
iPR	3.8	3.2	1.9	2.4	2.6	2.7	83.0	89.0	88.9
iP9G	0.4	1.6	1.0	4.9	4.5	4.2	99.1	87.4	93.3
Z	3.4	1.1	0.6	6.8	2.8	1.8	115.5	102.2	98.4
ZR	6.3	2.9	1.9	16.1	7.2	2.5	91.0	72.8	88.6
Z9G	2.6	2.0	4.5	13.5	5.3	4.7	94.3	112.2	103.8
DHZ	11.1	4.7	4.8	5.7	3.9	1.1	109.1	108.8	103.6
DHZR	6.9	3.5	5.4	3.6	0.04	7.4	105.8	104.8	93.6

Table 6
Amounts of endogenous CKs in *O. sativa* roots and leaves and *A. thaliana* seedlings (unit: ng g^{-1} F.W.).

Analyte	*O. sativa* roots	*O. sativa* leaves	*A. thaliana*
iP	0.22 ± 0.01	0.39 ± 0.01	n.d.
iPR	0.91 ± 0.03	0.29 ± 0.05	3.17 ± 0.11
iP9G	0.75 ± 0.02	0.62 ± 0.02	1.03 ± 0.10
Z	n.d.	0.22 ± 0.01	0.16 ± 0.01
ZR	2.65 ± 0.02	1.82 ± 0.40	1.44 ± 0.17
Z9G	0.82 ± 0.09	0.26 ± 0.02	1.08 ± 0.04
DHZ	0.32 ± 0.06	n.d	n.d.
DHZR	n.d.	n.d.	0.11 ± 0.01

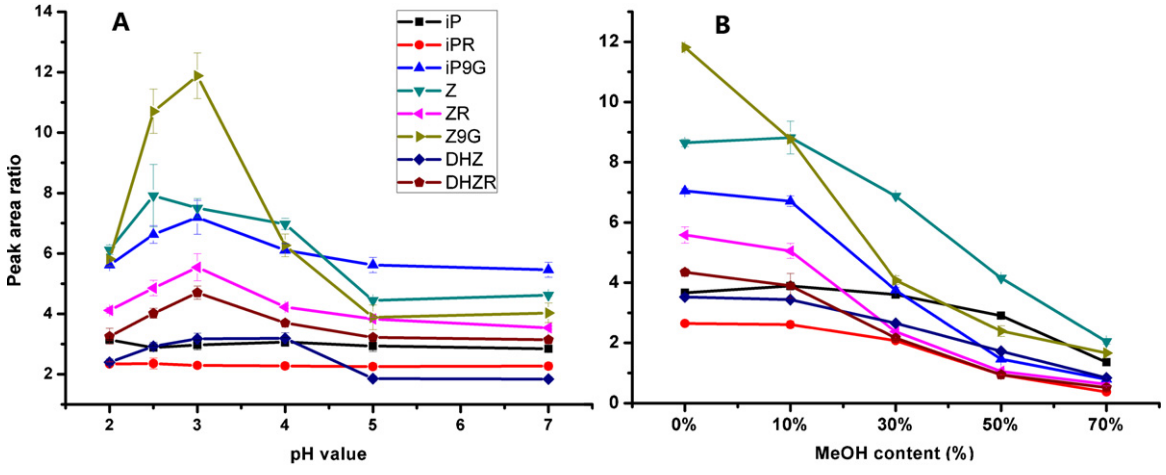

Fig. 5. The effect of matrix pH (A) and MeOH content (B) on extraction efficiency. Cytokinins standards were spiked in sample solutions at 1 ng mL^{-1}.

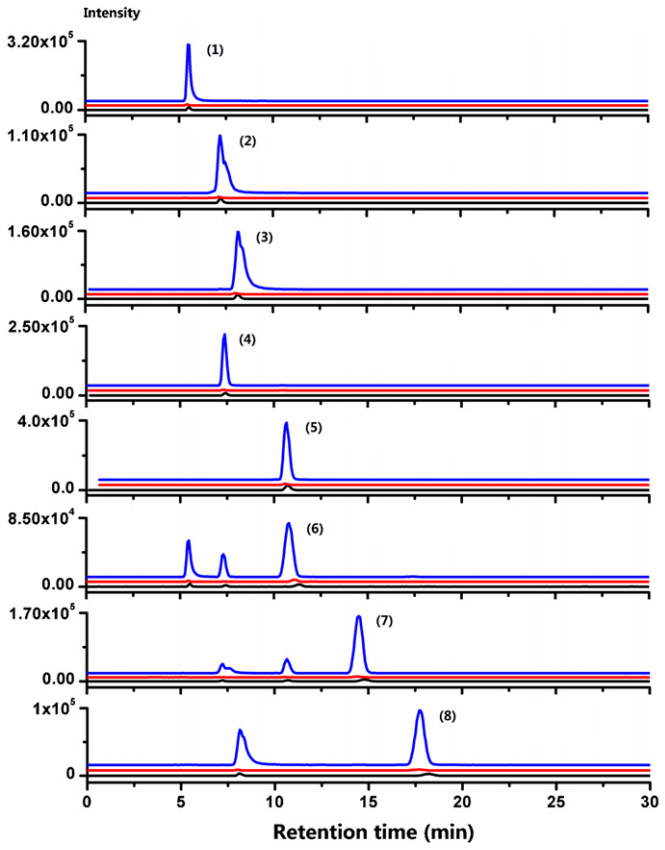

Fig. 6. The MRM chromatograms of directed HILIC–MS/MS analysis of CKs (lower), and CKs extracted by MPS-modified Fe_3O_4/SiO_2 (middle) and Fe_3O_4/SiO_2/P(AMPS-co-EGDMA) (upper). Peaks: (1) iPR; (2) t-ZR; (3) DHZR; (4) iP9G; (5) Z9G; (6) iP; (7) Z; (8) DHZ.

and 50 ng g^{-1}). Data in Table 5 showed that the relative recoveries of eight CKs were between 72.8 and 115.5%. The intra- and inter-day precisions for recoveries of eight CKs were less than 11.1% and 16.1%, respectively. The results indicated that the accuracy and precision of the current method are satisfactory for the determination of CKs in plant samples.

3.6. Quantification of CKs in O. sativa roots, leaves and A. thaliana seedlings

In the past decade, many studies have drawn their attention to the role of cytokinins in root development. For example, CKs can positively control the vascular differentiation of root meristem [34], influence cell proliferation in emerging crown root primordial [35], and can also negatively regulate the lateral root formation and root apical meristem activity [36–38]. Some scientist described that the role of CKs in root meristem maintenance may be the best characterized molecular mechanism of cytokinin action [4], indicating the importance of CKs in plant roots.

In this case, the endogenous levels of eight CKs in *O. sativa* roots were determined by the developed MSPE–HILIC–MS/MS method under the above optimized conditions. Fig. 7 shows the MRM chromatograms of detected CKs in *O. sativa* roots obtained by MSPE/HILIC/ESI-MS/MS method. Then, we applied the method to other plant samples, and the amounts of endogenous CKs in *O. sativa* roots, leaves and *A. thaliana* seedlings are listed in Table 6. It can be seen that the major CKs: iP, iPR, iP9G, Z, ZR, Z9G, DHZ and DHZR can be sensitively detected and determined from not only *O. sativa* roots but also other plant samples like *O. sativa* leaves and *A. thaliana* seedlings.

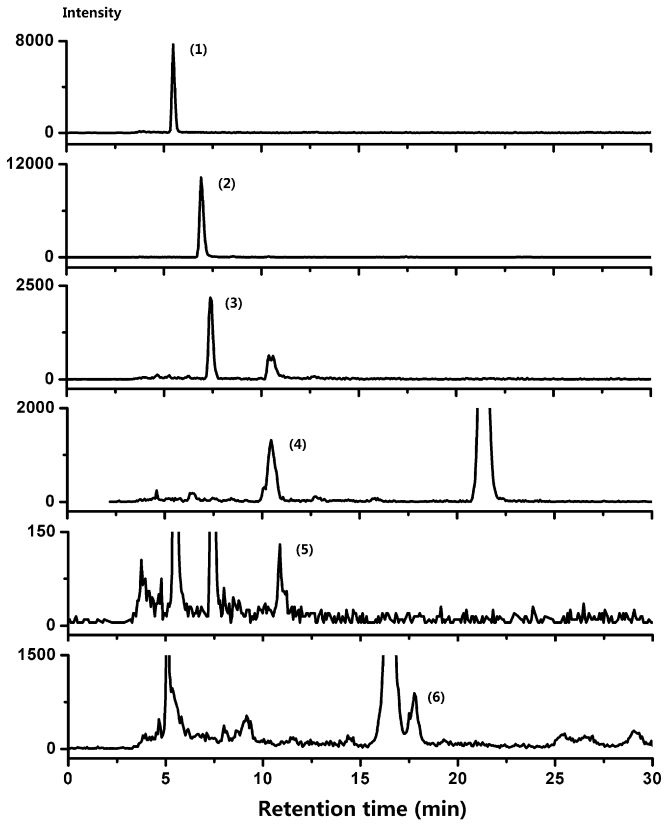

Fig. 7. The MRM chromatograms detected endogenous CKs in *O. sativa* roots. Peaks: (1) iPR; (2) t-ZR; (3) iP9G; (4) Z9G; (5) iP; (6) DHZ.

4. Conclusion

In this study, a new magnetic porous polymer, Fe_3O_4/SiO_2/P(AMPS-co-EGDMA), was successful prepared and used as an extraction medium for MSPE. The polymer has a large specific surface and a porous structure. The preparation exhibited excellent reproducibility, which made it a promising material for MSPE. Through a series of investigation, the magnetic polymer sorbent was demonstrated to show good extraction capacity towards CKs in plant samples. By combination with HILIC–MS/MS, a rapid, simple, and effective analytical method (MSPE–HILIC–MS/MS) for the analysis of CKs in *O. sativa* roots, leaves and *A. thaliana* seedlings was established.

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References

[1] C.O. Miller, F. Skoog, M.H.V. Saltza, F.M. Strong, J. Am. Chem. Soc. 77 (1955) 1392.
[2] K. Hoyerová, A. Gaudinová, J. Malbeck, P.I. Dobrev, T. Kocábek, B. Šolcová, A. Trávníčková, M. Kamínek, Phytochemistry 67 (2006) 1151.
[3] F. Skoog, C.O. Miller, Symp. Soc. Exp. Biol. 11 (1957) 118.
[4] S. Perilli, L. Moubayidin, S. Sabatini, Curr. Opin. Plant Biol. 13 (2010) 21.
[5] S.H. Howell, S. Lall, P. Che, Trends Plant Sci. 8 (2003) 453.
[6] O.N. Kulaeva, N.N. Karavaiko, S.Y. Selivankina, I.E. Moshkov, G.V. Novikona, Y.V. Zemlyachenko, S.V. Shipilova, E.M. Orudgev, Plant Growth Regul. 18 (1996) 29.
[7] P.O. Björkman, E. Tillberg, Phytochem. Anal. 7 (1996) 57.

[8] M. Kojima, T.K. Nobusada, H. Komatsu, K. Takei, T. Kuroha, M. Mizutani, M. Ashikari, M. Ueguchi-Tanaka, M. Matsuoka, K. Suzuki, H. Sakakibara, Plant Cell Physiol. 50 (2009) 1201.
[9] Z. Liu, F. Wei, Y.Q. Feng, Anal. Methods 2 (2010) 1676.
[10] X.Q. Pan, R. Welti, X.M. Wang, Nat. Prot. 6 (2010) 986.
[11] O. Novak, E. Hauserova, P. Amakorova, K. Kolezal, M. Strnad, Phytochemistry 69 (2008) 2214.
[12] L.Y. Ge, J.W.H. Yong, S.N. Tan, E.S. Ong, Electrophoresis 27 (2006) 2171.
[13] O. Novak, P. Tarkowski, D. Tarkowska, K. Dolezal, R. Lenobel, M. Strand, Anal. Chim. Acta 480 (2003) 207.
[14] R. Horgan, in: J.R. Hillman (Ed.), Isolation of Plant Growth Substances, Society for Experimental Biology, Seminar Series 4, Cambridge University Press, Cambridge, UK, 1978, p. 97.
[15] P.I. Dobrev, M. Kaminek, J. Chromatogr. A 950 (2002) 21.
[16] Y. Izumi, A. Okazawa, T. Bamba, A. Kobayashi, E. Fukusaki, Anal. Chim. Acta 648 (2009) 215.
[17] A.G. Oomen, P. Mayer, J. Tools, Anal. Chem. 72 (2000) 2802.
[18] Z. Liu, B.F. Yuan, Y.Q. Feng, Phychem. Analysis, doi:10.1002/pca.2353.
[19] Q. Gao, D. Luo, J. Ding, Y.Q. Feng, J. Chromatogr. A 1217 (2010) 5602.
[20] L. Sun, X. Sun, X.B. Du, Y.S. Yue, L.G. Chen, H.Y. Xu, Q.L. Zeng, H. Wang, L. Ding, Anal. Chim. Acta 665 (2010) 185.
[21] Q. Gao, D. Luo, M. Bai, Z.W. Chen, Y.Q. Feng, J. Agric. Food Chem. 59 (2011) 8543.
[22] I.I. Sukhanova, M.A. Dikunets, E.D. Viryus, G.M. Rodchenkov, J. Anal. Chem. 66 (2011) 807.
[23] X.L. Zhang, H.Y. Niu, Y.Y. Pan, Y.L. Shi, Y.Q. Cai, J. Colloid Interface Sci. 362 (2011) 107.
[24] Y.R. Song, S.L. Zhao, P. Tchounwou, Y.M. Liu, J. Chromatogr. A 1166 (2007) 79.
[25] X.L. Zhao, Y.L. Shi, T. Wang, Y.Q. Cai, G.B. Jiang, J. Chromatogr. A 1188 (2008) 140.
[26] J. Ding, Q. Gao, D. Luo, Z.G. Shi, Y.Q. Feng, J. Chromatogr. A 1217 (2010) 7351.
[27] X. Zhang, H. Niu, Y. Pan, Y. Shi, Y. Cai, Anal. Chem. 82 (2010) 2363–2371.
[28] J.H. Jiang, H.B. Lim, Microchem. J. 94 (2010) 148.
[29] D.W. Qi, Y. Mao, J. Lu, C.H. Deng, X.M. Zhang, J. Chromatogr. A 1217 (2010) 2606.
[30] S.J. Guo, D. Li, L.X. Zhang, J. Li, E.K. Wang, Biomaterials 30 (2009) 1881.
[31] G.Y. Liu, H. Wang, X.L. Yang, Polymer 50 (2009) 2578.
[32] Q. Ma, X.Z. Hu, J.C. Huang, Y.Q. Feng, Chin. J. Chromatogr. 27 (2009) 731.
[33] X.T. Peng, Z.G. Shi, Y.Q. Feng, Food Anal. Methods 4 (2011) 381.
[34] T. Werner, T. Schmülling, Curr. Opin. Plant Biol. 12 (2009) 527.
[35] Y. Zhao, Y. Hu, M. Dai, L. Huang, D.X. Zhou, Plant Cell 21 (2009) 736.
[36] T. Werner, V. Motyka, V. Laucou, R. Smets, H.V. Onckelen, T. Schmülling, Plant Cell 15 (2003) 2532.
[37] T. Werner, V. Motyka, M. Strnad, T. Schmülling, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 10487.
[38] M. Riefler, O. Novak, M. Strnad, T. Schmülling, Plant Cell 18 (2006) 40.